REMARKS/ARGUMENTS

In response to the Office Action of March 9, 2004, Applicants request re-examination and reconsideration of this application for patent pursuant to 35 U.S.C. 132.

Claim Status/Support for Amendments

Claims 1, 39 and 44-46 have been amended. Claims 2-38 were cancelled in a previous reply (filed on September 22, 2003). Claims 39-46 are withdrawn from consideration. It is understood that the remaining claims (39-46), drawn to a non-elected invention, will remain pending, albeit withdrawn from consideration on the merits at this time. Claim 1 is under examination. Claims 1 and 39-46 remain pending in the instant application.

No new matter has been added by the amendments to the specification made herein.

In the "Background of the Invention" section a punctuation error (parentheses were closed) was corrected at page 1, line 23.

The disclosure of prior art, PCT/EP97/04396, at page 5 has been amended to correct a typographical error in the international application number. The corresponding international publication number has also been added.

The "Description of the Figures" section has been amended to

add sequence identification numbers and clearly indicate that Figures 2 and 4-8 show the mass spectrum profiles of the disclosed peptides. The term "specimen" was also deleted from the description of Figures 1 and 3.

Several protocols at pages 41-45 have been amended to properly identify trademark names (SEPHAROSE, TRITON, TRIS and EPPENDORF). The protocol titles at page 41 (line 16), page 42 (lines 7 and 22), page 43 (line 13) and page 44 (line 3) were underlined in the original disclosure and do not indicate text amended in the instant Response.

In the "Detailed Description" section, the term "cerebrospinal fluid" has been added to define the abbreviation "CSF" at page 49, line 23 in order to provide explicit support for cerebrospinal fluid as recited in claim 41. "CSF" is a well known abbreviation for cerebrospinal fluid in the biochemical art. A typographical error within the same paragraph has also been amended (skill replaced skilled).

No new matter has been added by the amendments to the claims made herein.

Claim 1 has been amended to specifically claim the biopolymer markers (SEQ ID NO:1 and SEQ ID NO:4). The term "biopolymer marker" is used throughout the originally filed specification, see, for example, page 1, line 8.

Claim 39 has been amended to more clearly disclose the relationship between the presence of the claimed biopolymer markers (SEQ ID NO:1 and SEQ ID NO:4) and Type II diabetes. Claim 39 has also been amended to clearly indicate how the presence of the claimed biopolymer markers is determined from mass spectrum profiles. The changes to claim 39 find basis throughout the original disclosure, see, for example, page 35, lines 14-18, page 46, line 14 to page 47, line 2 and Figures 1-4.

Claim 44 has been amended to correspond with the biopolymer markers of claim 1, as amended herein. Support for various types of kits can be found in the original disclosure, see, for example, page 36, lines 9-12 and page 47, line 21 to page 49, line 7.

Claims 45 and 46 were amended to provide proper antecedent basis for the term "kit" in claim 44, as amended herein.

As used herein, the term "normal patient" or "healthy patient" refers to a patient who is normal and/or healthy with regard to Type II diabetes, but may or may not have other conditions which are not tested.

Oath/Declaration

Applicants note that while the signed Declaration filed on March 5, 2002 contains the signature of Dr. John Marshall (inventor 2), the date of signature is omitted.

Applicants herewith submit a newly-executed oath or declaration as required.

Restriction

New claims 39-46 were added in the Response filed on September 22, 2003. The Examiner has withdrawn claims 39-46 from consideration on the merits at this time as being directed to a non-elected invention.

Request for Rejoining of Claims

The Examiner states that Applicants' argument of the decision in *In re Ochiai* is noted but is not deemed persuasive, as PTO practice in view of that decision is directed to rejoinder of claims after allowable subject matter has been indicated, and not to withdrawl of restriction requirements.

Applicants submit that the request for rejoining of claims was not made as an argument for withdrawal of the restriction requirement. Page 12 of the Reply (filed on September 22, 2003) clearly states, "...Applicants respectfully request that the Examiner enter new claims 39-46 in the instant application as being drawn to a non-elected invention and **consider joining** them (claims 39-46) with claim 1 of the elected invention (Group I) **upon the**

Examiner's determination that claim 1 of the elected invention is allowable ...". The request for rejoining claims is repeated herein for the convenience of the Examiner.

Considering that claims 39-46 are limited to the use of an isolated biopolymer marker selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:4, a search of these claims would encompass these specific biopolymer markers. The instant application is related in claim format to several other applications, both pending and issued, of which serial number 09/846,352 is exemplary. In an effort to maintain equivalent scope in all of these applications, Applicants respectfully request that the Examiner consider rejoining claims 39-46 in the instant application, which are currently drawn to non-elected inventions, under the decision in *In re Ochiai* (MPEP 2116.01) with claim 1 of the elected invention, upon the Examiner's determination that the claim of the elected invention is allowable and in light of the overlapping search. If the biopolymer markers of SEQ ID NO:1 and SEQ ID NO:4 are found to be novel, methods and kits limited to their use should also be found novel.

Rejection under 35 USC 112

Claim 1, as presented on September 22, 2003, stands rejected under 35 USC 112, first paragraph, as containing subject matter

which allegedly was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

As pointed out by the Examiner, the reference to MPEP 2165.03 at page 17 of the Reply filed on September 22, 2003, is misplaced and should not be construed to support the statement afer which it is quoted.

The Examiner continues to maintain the position that the instant specification, as filed, fails to provide any evidence or sound scientific reasoning that would support a conclusion that an isolated biopolymer marker selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:4 could be used in a diagnosis of Type II diabetes.

Applicants respectfully disagree with the Examiner's position.

Although Applicants believe that the instant specification fully supports the claim that an isolated biopolymer marker selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:4 is diagnostic for Type II diabetes, in the interest of compact, efficient prosecution Applicants have amended the claims to recite that the isolated biopolymer markers are linked to Type II diabetes.

According to the web site dictionary.com the term "linked"

refers to the condition of being associated with or connected to (see attached document as accessed from the internet; reference 1). The instant specification fully supports a connection and/or an association of the claimed peptides with Type II diabetes. The instant specification states at page 35, lines 14-18 that an objective of the invention is to evaluate samples containing a plurality of biopolymers for the presence of disease specific marker sequences which evidence a link to at least one specific disease state.

Claim 1 has been amended to specifically recite an isolated biopolymer marker selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:4. Claim 1, as amended herein, does not recite that the claimed isolated biopolymer markers are diagnostic for Type II diabetes, nor does it recite that the claimed isolated biopolymer markers are related to Type II diabetes, even though Applicants believe that the instant specification as originally filed fully supports both of these recitations. Furthermore, the phrase "consisting of" is closed language and excludes any element, step or ingredient not specified in the claim (see MPEP 2111.03). Thus, the scope of claim 1 is limited to these specific biopolymer markers (SEQ ID NOS:1 and 4).

The Examiner is respectfully reminded that all questions of enablement are evaluated against the claimed subject matter (see

MPEP 2164.08)and further that the claimed subject matter is interpreted in light of the specification. Thus, in the instant case, the specification should enable the evaluation of samples containing a plurality of biopolymer markers for the presence of disease specific marker sequences which evidence a link to at least one specific disease state; specifically the markers sequences SEQ ID NOS:1 and 4 evidence a link to Type II diabetes.

It has been established that in order to comply with the enablement requirement all that is necessary is that one skilled in the art be able to practice the claimed invention, given the level of knowledge and skill in the art (see MPEP 2164.08).

Applicants assert that those of skill in the art are both highly knowledgeable and skilled, such that it is obvious that no undue experimentation would be required for a skilled artisan to follow any of the electrophoretic, chromatographic and mass spectrometric protocols presented in the instant specification in order to use the claimed invention. The Examiner agrees with Applicants' assertion (page 4, final office action mailed on March 9, 2004). One of skill in the art would be able to view a gel, such as those shown in Figures 1 and 3 from which the claimed biopolymer markers were identified, and recognize a difference between two comparable states (disease state vs. non-disease state) and further recognize that the peptides present within the gel are

differentially expressed between the two sample types. Moreover, one of ordinary skill in the art would be able to compare the established mass spectrum profile of an isolated biopolymer marker selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:4 to a mass spectrum profile obtained from a patient's sample to confirm the presence or absence of a peak at about 1630 daltons, wherein the presence of the claimed biopolymer markers is indicative of a link to Type II diabetes.

The Examiner provides an analysis of the data presented in Figure 1 which she asserts appears to be in conflict with Applicants' previous statements regarding the data. The Examiner asserts that Figure 1 is a photograph of a gel containing 10 standard lanes; lanes 1-4 are normal samples, lanes 5-8 are Type II diabetes samples, lane 9 appears to be a another control or is designated for molecular weight standards.

Applicants respectfully submit that the Examiner's assertion regarding lane 9 of Figure 1 is incorrect. Lane 9 is not a control nor is it reserved for molecular weight standards, but rather, it contains a sample from a Type II diabetes patient. Lane 10 is reserved for the molecular weight standards. Lane 9 was disclosed as containing a sample obtained from a Type II diabetes patient at page 19 of the previous response filed on September 22, 2003.

The Examiner further asserts that analysis of Band 1 in Figure

3 appears to be complicated because Band 1 is practically indistinguishable in normal control samples.

Applicants respectfully disagree with the Examiner's assertion and submit that Band 1 is clearly indicated in lane 9 of Figure 3 and is also seen in lanes 7 and 8. Lanes 7-9 of Figure 3 all contain samples obtained from normal control patients.

The "test of enablement" is whether one reasonably skilled in the art could make and/or use the invention from the disclosures in the patent combined with information known in the prior art without undue experimentation (see MPEP 2164.01).

Furthermore, the decision in *In re Brandstader* (179 USPQ 286; MPEP 2164.01) has established that the evidence provided by applicant (to overcome an enablement rejection) need not be conclusive but merely convincing to one of skill in the art.

Applicants respectfully submit that the instant specification provides sufficient evidence to convince one of skill in the art that the claimed peptides (SEQ ID NOS:1 and 4) are linked to and/or associated with Type II diabetes.

Claim 1 has been amended to specifically recite an isolated peptide selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:4; peptides that the instant specification identifies as related to Type II diabetes (see page 46, line 14 to page 47, line 2). Claim 1, as amended herein, does not recite that the claimed

isolated biopolymer markers are diagnostic for Type II diabetes, nor does it recite that the claimed isolated biopolymer markers are related to Type II diabetes, even though Applicants believe that the instant specification as originally filed fully supports both of these recitations. Furthermore, the phrase "consisting of" is closed language and excludes any element, step or ingredient not specified in the claim (see MPEP 2111.03). Thus, the scope of claim 1 is limited to these specific biopolymer markers (SEQ ID NOS:1 and 4).

The specification provides information regarding the claimed peptides; for example, at page 46, line 14 to page 47, line 2, SEQ ID NO:1 is identified as having a molecular weight of about 1630 daltons. The description of Figure 2 at page 37 indicates that the spectra depicted in the figure is that of an ion having a molecular weight of about 1630 daltons. The spectra shown in Figure 2 was obtained from Band 1 as shown in the gels of Figures 1 and 3. The descriptions of the figures have been amended to clarify that the data shown in the figures is representative of the claimed peptides (SEQ ID NOS:1 and 4) and the other disclosed peptides.

Figures 1 and 3 demonstrate that the claimed biopolymer markers show increased intensity in samples obtained from control patients who were healthy in regard to Type II diabetes when compared with samples obtained from patients with a history of Type

II diabetes. Thus, a difference is seen between two comparable samples (disease versus non-disease), suggesting that the differentially expressed peptides are linked to the disease state; in this instance, SEQ ID NOS: 1 and 4 are linked to Type II diabetes.

The Examiner asserts that the specification, as originally filed, does not provide a precise protocol on how to analyze the data obtained by carrying out the disclosed protocol.

Applicants respectfully disagree with the Examiner's assertion.

Applicants believe that the instant specification, as originally filed, discloses a precise protocol that one of skill in the art can carry out in order to practice the disclosed methods successfully and interpret the results. The data is analyzed by comparing the mass spectrometric profile of a disease sample to the mass spectrometric profile of a control sample. Page 5 ,line 9 to page 6, line 3 of the instant specification discloses a general outline of how to analyze the data obtained by carrying out the disclosed methods. Page 26, lines 6-13 of the instant specification further describes how samples were compared to develop data and indicates how peptides were selected as notable sequences. This passage also discloses how certain peptides were selected from a plurality of molecules found within a sample and how peptides were

deemed to be evidentiary of a disease state. Page 47, lines 10-12 of the instant specification clearly states that the steps of the invention include obtaining a sample from a patient and conducting an MS (mass spectrometry) analysis on the sample. One of ordinary skill in the art would know how to interpret the data gathered from a mass spectrometric profile.

Thus, Applicants respectfully submit that the instant specification, contrary to the Examiner's assertion, does provide an explanation of how to analyze data obtained by carrying out the disclosed methods.

Furthermore, even if the specification did not provide such explanation, the method for analysis of data used by the instant inventors is commonly practiced in the art (proteomics).

For example, Lubec et al. (see attached abstract Journal Neural Transmission Supplement 57:161-177 1999; reference 2) disclose an experiment in which proteomic techniques, specifically electrophoresis and mass spectrometry, were carried out to detect differences in protein expression between Down's syndrome patients, Alzheimer's patients and "normal" control patients. In a manner similar to that of the instant inventors, Lubec et al. analyzed the increase and/or decrease in expression of a particular protein (DRP-2) when hypothesizing about the neuropathological findings in Alzheimer's disease and Down's syndrome.

Additionally, Oppermann et al. (European Journal of Biochemistry 267(15):4713-4719 2000; reference 3) not only discloses electrophoretic and mass spectrometric experiments very similar to the instantly described experiments; Oppermann *et al.* also analyze their data as the data is analyzed by the instant inventors. Oppermann et al. compared protein expression patterns in brain tissue from patients with and without(control) chromosome 21 trisomy (Down's syndrome)and identified truncated structural proteins (tubulin, actin) in trisomy samples which were absent from the control samples. Oppermann et al. interpreted this absence to indicate an increase in proteolysis in Down's syndrome.

The data presented in the figures, derived from the working examples, discloses that the claimed peptides (SEQ ID NOS:1 and 4) are differentially expressed between Type II diabetes and healthy controls, thus it can be reasonably predicted that such peptides are linked to Type II diabetes. Furthermore, the figures identify SEQ ID NOS:1 and 4 and the specification discloses how such sequences were identified as notable sequences in relation to Type II diabetes.

Thus, Applicants contend that a skilled practitioner would find that the data presented in the instant specification is convincing with regard to a link between the claimed biopolymer marker peptides (SEQ ID NOS:1 and 4) and Type II diabetes.

Considering the above comments, it is clear that both the specification and the prior art disclose how to analyze data obtained from carrying out the disclosed protocols. Accordingly, Applicants respectfully contend that the instant invention passes the "test of enablement" since one skilled in the art could use the invention from the disclosures in the specification coupled with information known in the prior art without undue experimentation.

The Examiner asserts that the Declaration of Jackowski under 37 CFR 1.132 filed on September 22, 2003 is insufficient to overcome the rejection of claim 1. The Examiner further asserts that in view of the absence of information of the presence or absence of peptides of SEQ ID NOS:1 and 4 in normal controls, and most importantly, total absence of working examples of factual diagnosis of Type II diabetes using the disclosed protocol, such as cases when peptides of SEQ ID NOS:1 and 4 were found in samples of patients, which were further diagnosed with Type II diabetes (positive control), and not found in samples of patients diagnosed with other diseases or pathological conditions (negative control), a skilled artisan would have to resort to a substantial amount of undue experimentation to discover how to use the claimed biopolymer markers of SEQ ID NOS:1 and 4 in the diagnosis of Type II diabetes.

Applicants respectfully disagree with the Examiner's assertions.

Applicants believe that the instant specification fully enables the use of the claimed biopolymer markers for the diagnosis of Type II diabetes; however, in order to further prosecution, the claims have been amended such that the biopolymer markers are linked to Type II diabetes. Thus, the claims no longer recite that the claimed biopolymer markers are diagnostic for Type II diabetes.

The purpose of the Declaration of Jackowski under 37 CFR 1.132 filed on September 22, 2003 was to provide clarification of the use of controls in the experiments disclosed in the instant specification. This fact was already established by the Applicants at page 20 of the previous Response also filed on September 22, 2003.

The gels pictured in both Figures 1 and 3 clearly show a comparison between samples obtained from normal patients and samples obtained from Type II diabetes patients. The fibronectin precursor of Band 1 is seen more intensely (in both gels) in lanes displaying samples obtained from normal patients. Thus, Applicants respectfully submit that the Examiner's statement regarding the absence of information of the absence or presence of peptides of SEQ ID NOS:1 and 4 in normal controls is incorrect.

Additionally, Figures 1 and 3 show data obtained from experiments wherein two physiological states were compared; normal and Type II diabetes. These experiments were carried out using the

disclosed protocol and thus constitute working examples. Thus, Applicants respectfully submit that the Examiner's statement regarding the total absence of working examples of factual diagnosis of Type II diabetes using the disclosed protocol is also incorrect.

The Examiner states that it appears that in order to use the claimed peptides for diagnosis of Type II diabetes, samples must already be identified as normal or Type II diabetes samples.

Applicants respectfully disagree with the Examiner's statement.

The experiments disclosed in the instant specification establish mass spectrum profiles with regard to the claimed biopolymer markers which are intended to be used as reference points for comparison to an unknown sample in the diagnostic assay contemplated by the instant inventors. The mass spectrum profiles of the claimed biopolymer markers (SEQ ID NOS:1 and 4) are characterized by a peak at around 1630 (see page 46, lines 14-21 and Figures 2 and 4 of the instant specification). These known mass spectrometric profiles of the claimed biopolymer markers are compared with the mass spectrometric profile of the unknown sample in the diagnostic assay contemplated by the instant inventors.

When an unknown sample is presented for analysis, the sample is resolved by polyacrylamide gel electrophoresis. If the

fibronectin protein (Band 1) appears in the unknown sample, at this point one could hypothesize that the fibronectin precursor protein has not been damaged by a disease process and thus, the unknown sample is likely to be normal with respect to Type II diabetes.

After excision from the gel, the proteins from the unknown sample are purified (from the gel) and subjected to enzymatic digestion, chromatography and identification by mass spectrometry techniques. The mass spectra produced from the unknown sample is compared with the established spectra, both normal and diseased. If the mass spectrum profiles of the claimed peptides are not found in the unknown sample, but instead the profile of the whole protein is seen, similar to the expression established in the normal controls, Type II diabetes is indicated to be absent. Alternatively, if the mass spectrum profile of the patient sample displays the established mass spectrometric profiles of the claimed biopolymer markers (a peak at around 1630 Daltons) this indicates a link to Type II diabetes.

Thus, Applicants respectfully submit that the Examiner's statement regarding "unknown" samples which were already identified is incorrect.

The Examiner continues to assert that the association of the instant peptides with Type II diabetes is not supported by any

evidence of record and further contends that the art does not recognize a specific association of the claimed biopolymer markers (SEQ ID NOS:1 and 4, which are fragments of the fibronectin precursor protein) with Type II diabetes.

Applicants respectfully disagree with the Examiner and contend that one of skill in the art would understand that the claimed biopolymer markers are linked to Type II diabetes.

The guidelines for a "test of enablement" indicate that if a statement of utility in the specification contains within it a connotation of how to use, and/or the art recognizes that standard modes of administration are known and contemplated, 35 USC 112 is satisfied.

Additionally, it has been established that the mere fact that something has not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it (see MPEP 2164.02).

The instant specification discloses biopolymer markers (SEQ ID NOS:1 and 4) which are linked to Type II diabetes. The data derived from the working examples and presented in Figures 1 and 3 evidences a link between the claimed peptides and Type II diabetes.

The claimed biopolymer markers (SEQ ID NOS:1 and 4) were found to be differentially expressed between Type II diabetes and normal

controls through use of proteomics techniques. The field of proteomics was established in the mid 1990's, is well-studied and is expanding rapidly to advance medical diagnostics and therapeutics. In proteomics research, differential protein expression patterns between normal and diseased cells are routinely analyzed and compared (see attached press release form Scimagix, Inc. as accessed from the internet; reference 4).

Expression proteomics is the large-scale analysis of protein expression and function in which the goal is to detect and identify all or a subset of the proteins present in a particular sample and find out which of these proteins are present, absent and/or differentially expressed in a related sample subject to a specific variation (see attached document GIGA Proteomics Facility, Belgium, as accessed from the internet; reference 5). Proteomics techniques make it possible for researchers to immediately highlight proteins that are differentially abundant in one state vs. another (for example, tumor vs. normal or before and after a treatment; see attached article, Liotta et al. Breast Cancer Research 2:13-14 1999 as accessed from the internet, page 2, second paragraph; reference 6). A protein found only in a diseased sample may prove to be a useful drug target or diagnostic marker (see attached document GIGA Proteomics Facility, Belgium, as accessed from the internet; reference 5).

The claimed biopolymer markers are identified as fragments of the fibronectin precursor protein at page 46, lines 14-21.

The Examiner alleges that there is an absence of art recognition of any specific association of the claimed biopolymer markers (SEQ ID NOS:1 and 4), which are fragments of the fibronection precursor protein, with Type II diabetes.

Applicants respectfully disagree with the Examiner's allegation and respectfully submit that the allegation is insufficient to support the enablement rejection, since it has been established that the fact that something has not previously been done is not a sufficient basis for rejection.

However, while the art does not recognize an association between the specifically claimed peptides (SEQ ID NOS: 1 and 4) and Type II diabetes, the art does recognize, contrary to the Examiner's allegation, an association between fibronectin and Type II diabetes.

Fibronectin is a key component of the extracellular matrix; functioning, through a series of binding domains, to maintain normal cell morphology via organization of cell attachment to the extracellular matrix. Fibronectin is particularly prone to fragmentation since the regions between the binding domains are highly susceptible to proteolysis. Fibronectin fragments are known to have functions not found in the intact protein, such as exerting

affects on the proliferation and migration of endothelial cells (see attached article of Grant et al. Diabetes 47:1335-1340 1998; reference 7 which contains the information about fibronectin disclosed in the instant paragraph).

Additionally, increased proteolysis is known to contribute to the pathologic process of Type II diabetes (Comment by Luc Tappy on Gastadelli et al. Diabetes 49:1367-1373 2000; as accessed from the internet; reference 8).

Furthermore, excess fibronectin produced in diabetes is theorized to be available for fragmentation (Grant et al. Diabetes 47:1335-1340 1998; reference 7). Grant et al. (Diabetes 47:1335-1340 1998; reference 7)hypothesized that the formation of abnormal fibronectin fragments *in vivo* could facilitate aberrant angiogenesis, as seen in such conditions as proliferative diabetic retinopathy.

The instant inventors hypothesize that the stronger expression of the fibronection precursor in patients considered to be normal with regard to Type II diabetes when compared to expression seen in patients with a history of Type II diabetes indicates the fragmentation of fibronectin that may occur during the diabetic disease process.

Considering that there is a known increase in proteolysis in Type II diabetes and that fibronectin is particularly sensitive to

such proteolysis (degradation into fragments) and further considering the suggestion in the art that fibronectin fragments may be involved in diabetic processes such as proliferative retinopathy, a skilled artisan would find the hypothesis and data disclosed in the instant application entirely plausible, and thus would reasonably link the claimed biopolymer markers (SEQ ID NOS: 1 and 4) with Type II diabetes.

In conclusion, Applicants assert that the claimed fibronectin fragments (SEQ ID NOS:1 and 4) are indicative of a link to Type II diabetes; a statement which is enabled by the instant specification, as evidenced by the arguments presented herein. Applicants further assert that one of ordinary skill in the art when reviewing the instant specification, given the level of knowledge and skill in the art, would recognize how to use the claimed biopolymer markers as markers for Type II diabetes. Thus, Applicants respectfully request that this rejection under 35 USC 112, first paragraph now be withdrawn.

CONCLUSION

In light of the foregoing remarks, amendments to the specification, and amendments to the claims, it is respectfully submitted that the Examiner will now find the claims of the application allowable. Favorable reconsideration of the application is courteously requested.

Respectfully submitted,

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Examiner 109 reference 1

link[1] Pronunciation Key (lĭngk)
n.

1. One of the rings or loops forming a chain.
2.
   a. A unit in a connected series of units: *links of sausage; one link in a molecular chain.*
   b. A unit in a transportation or communications system.
   c. A connecting element; a tie or bond: *grandparents, our link with the past.*
3.
   a. An association; a relationship: *The Alumnae Association is my link to the school's present administration.*
   b. A causal, parallel, or reciprocal relationship; a correlation: *Researchers have detected a link between smoking and heart disease.*
4. A cuff link.
5. *Abbr.* **li** A unit of length used in surveying, equal to 0.01 chain, 7.92 inches, or about 20.12 centimeters.
6. A rod or lever transmitting motion in a machine.
7. *Computer Science.* A segment of text or a graphical item that serves as a cross-reference between parts of a hypertext document or between files or hypertext documents. Also called **hotlink, hyperlink**.

1: J Neural Transm Suppl. 1999;57:161-77.

Related Articles, Links

Expression of the dihydropyrimidinase related protein 2 (DRP-2) in Down syndrome and Alzheimer's disease brain is downregulated at the mRNA and dysregulated at the protein level.

Lubec G, Nonaka M, Krapfenbauer K, Gratzer M, Cairns N, Fountoulakis M.

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Deteriorated migration, axonal pathfinding and wiring of the brain is a main neuropathological feature of Down Syndrome (DS). Information on the underlying mechanisms is still limited, although basic functions of a series of growth factors, cell adhesion molecules, guidance factors and chemoattractants for brain histogenesis have been reported. We used proteomics to detect differences in protein expression between control, DS and Alzheimer's disease brains: In five individual brain regions of 9 individuals of each group we performed two dimensional electrophoresis with MALDI--identification of proteins and determined mRNA levels of DRP-2. Significantly decreased mRNA levels of DRP-2 in four brain regions of patients with DS but not with AD as compared to controls were detected. 2D electrophoresis revealed variable expression of DRP-2 proteins, which showed a high heterogeneity per se. Dysregulation of DRP-2 was found in brains of patients with DS and AD presenting with an inconsistent pattern, which in turn may reflect the inconsistent neuropathological findings in patients with DS and AD. The decrease of mRNA DRP-2 steady state levels in DS along with deteriorated protein expression of this repulsive guidance molecule of the semaphorin/collapsin family, may help to explain deranged migration and histogenesis of DS brain and wiring of AD brain.

PMID: 10666674 [PubMed - indexed for MEDLINE]

Examiner 109

reference 3

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1: Eur J Biochem. 2000 Aug;267(15):4713-9. Related Articles, Links

Full text

Identification of foetal brain proteins by two-dimensional gel electrophoresis and mass spectrometry comparison of samples from individuals with or without chromosome 21 trisomy.

Oppermann M, Cols N, Nyman T, Helin J, Saarinen J, Byman I, Toran N, Alaiya AA, Bergman T, Kalkkinen N, Gonzalez-Duarte R, Jornvall H.

Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden.

Protein expression in foetal brain with or without chromosome 21 trisomy (Down's syndrome) was analyzed by two-dimensional gel electrophoresis and mass spectrometry. Data generated by in-gel digestion and matrix-assisted laser desorption/ionization mass spectrometry allowed identification of 40 proteins. Most of these are common to syndrome and healthy subjects and represent different types of protein. However, a few proteins, identified as truncated structural proteins (tubulin, actin), were present in part of the trisomy samples but absent from the controls. This is interpreted to indicate increased proteolysis in the syndrome samples but could also reflect some altered expression or processing. Independent of the apparently increased proteolysis in the syndrome samples, and in spite of the use of total brain tissues, the results show that two-dimensional protein separation patterns are largely similar between the syndrome and control samples upon silver-staining, but that differences associated with structural components can be detected and identified.

PMID: 10903504 [PubMed - indexed for MEDLINE]

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Examiner 109
reference 4

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Scimagix and Compugen Sign Re-sale Agreement for 2D-Gel Analysis Product

Creating High-throughput 2D-Gel Analysis and Mining Products for Proteomics

REDWOOD SHORES, Calif., February 21, 2001 — Scimagix™ Inc. today announced an agreement for Scimagix to resell Compugen's Z3™ automated system for rapid analysis of 2-dimensional electrophoresis (2D) gels. The combination of Z3 with Scimagix's SIMS™ image data management system and ProteinMine™ 2D-gel analysis and mining application provides researchers with robust and accurate comparisons of 2D gels for differential protein expression and high-speed searching of similar gel expression patterns, and the ability to correlate 2D-gel data with other image or non-image data.

Under the agreement, Scimagix will resell Compugen's Z3 product worldwide together with Scimagix' SIMS product; both companies plan to integrate their products for seamless exchange of data. Financial details were not disclosed.

"Our agreement combines Compugen's extensive gel analysis methods with Scimagix's unique capability to search protein expression patterns," said Robert Dunkle, president and CEO of Scimagix. "Together we can offer the best approach for high-throughput 2D-gel analysis and mining, enabling researchers to derive faster and better insights from the information found in 2D gels. This high-throughput approach to analysis and mining of 2D gels is expected to accelerate proteomics research and drug discovery."

"Through our relationship with Scimagix we are able to offer customers a stronger proteomics solution, therefore serving the market in a more complete manner," said Dr. Michal Preminger, Vice President, Proteomics Business, Compugen. "Our agreement will expand the reach of both companies, providing innovative solutions for image data analysis and mining for pharmaceutical R&D."

Z3 Automated 2D-Gel Analysis
Compugen's Z3 product sets a benchmark for rapid and visually-oriented comparison of 2D gels to identify differentially expressed proteins, reducing the average overall analysis time per gel-pair to less than 15 minutes. In proteomics research, differential protein expression patterns between normal and diseased cells are routinely analyzed and compared. Z3 automates this process of comparative proteomics analysis to determine those proteins that are differentially expressed. Z3 is able to accomplish this through the use of complex image-analysis algorithms that rapidly and accurately align two gels, resulting in an easy-to-read visual analysis.

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About Compugen
Compugen (Nasdaq: CGEN) develops and markets platforms, tools and products that accelerate post-genomic research, the advanced study of proteins and protein pathways, and drug-target discovery. These products and services include: LEADS, Gencarta, DNA Chip design, Z3, LabOnWeb.com and Bioccelerators. Compugen's in-house molecular biology laboratories provide validation of its methodologies and also conduct original genomic and proteomic research. For additional information, please visit Compugen's corporate Web site at www.cgen.com and the company's Internet research engine for molecular biologists at www.LabOnWeb.com.

About Scimagix Inc.
Scimagix™ Inc., based in Redwood Shores, Calif., is a leading provider of image informatics solutions for the pharmaceutical and biotechnology industries. Its SIMS™ - Scientific Image Management System and ProteinMine™ 2D-gel analysis and mining application for proteomics research are the first in a new class of image retrieval, analysis and mining software that allows researchers to derive added value from images. Scimagix's products serve five of the top 15 pharmaceutical companies, including Pfizer Global R&D and Eli Lilly & Co. Unlocking the value of scientific images, image informatics promises to become an essential and pervasive technology for gaining new insights and direction for discovery research and development.

What is proteomics?

Examiner 0996
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The completion of the Human Genome Sequencing Project represents a major achievement in modern science. The wealth of information obtained through human genome analysis will certainly increase our knowledge of the cell biochemistry that defines the boundary between a healthy and a diseased individual. It will also contribute to the development of new tools for the diagnosis and treatment of human diseases.

Today, in terms of DNA sequences, scientists have in hand the complete genomes of a wide variety of organisms, spanning all forms of life from viruses, phages, archaea, and bacteria to eukaryotes.

Considering the size of the human genome (~3,200 Mb), an unexpectedly small number of human genes has been predicted: between 20,000 and 35,000 (the precise number is still the subject of much controversy). Genes make up less than 2 percent of the human DNA; the remaining DNA has important but as yet unknown functions that may include regulating genes and maintaining chromosome structure.

Direct access to the genome, however, is only a preliminary step towards understanding biological processes, because detecting all coding regions in a genome sequence remains a difficult task. This is especially true in eukaryotes, where current algorithms, although quite efficient, are unable to detect with certainty all exons, are ill-equipped to discriminate different splice variants, and are unable to identify small proteins (which are numerous and essential to many biological processes).

Even if we identify all potential protein coding regions in the human genome, we will still be missing some crucial information, because genomic information by itself does not allow efficient prediction of all the post-modifications observed in proteins.

Diverse mechanisms can result in the expression of many protein variants from the same gene locus in a single species: single nucleotide polymorphisms (SNP), gene splicing, alternative splicing of pre-mRNA, RNA editing, translational frame shifts and hopping, proteolytic cleavage of the protein (to eliminate signal sequences or to create transit peptides or pro-peptides) and post-translational modifications of amino acid residues which affect a vast majority of proteins (acetylation, phosphorylation, glycosylation, lipidation, etc - more than a hundred different types of PTMs are currently known).

Hence, the number of different protein molecules expressed by the human genome is probably closer to a million than to the hundred thousand generally considered by genome scientists.

The **"proteome"** can be defined as all proteins expressed by a cell at a particular time and under specific conditions. The aim of **"proteomics"** is to identify, characterise, and quantify all proteins involved in a particular pathway, organelle, cell, tissue, organ, or organism and that can be studied simultaneously in order to obtain accurate and comprehensive data about that system and to correlate expression-level changes and/or protein PTMs with growth conditions, the cell cycle stage, a disease state, external stimuli, levels of expression of other proteins, or other variables.

Why proteomics?

Proteomics has the potential to revolutionise the development of innovative clinical diagnostics and pharmaceutical therapeutics.

There are many reasons why understanding the proteome will be more useful than understanding the genome:

- Whereas every cell in an organism contains an identical copy of the complete set of genes necessary to build a functional individual, this set of genes is only a source of information, which must be expressed in order to function. In complex organisms, this information is used differently in different cells in order to produce different types of tissues, organs, or cells (i.e. liver, muscle, bone, neurons, blood cells...), and these differences are due to the proteins that exploit the genetic information differently in each cell.

What is proteomics?

- From these considerations it appears that the real actors behind the complexity of life-sustaining biochemical mechanisms are the proteins, with their intricate patterns of interactions with each other and with other biological molecules and their relations with the external environment.

- Whilst some protein polymorphisms are linked to disease states, most are not. Yet they do have in many cases a direct or indirect effect on the activities of the proteins concerned. For example, it is estimated that each human protein exists, on the average, in ten to fifteen different post-translationally modified forms, with - presumably - different functions. Much of the information processing in healthy and diseased human cells can be studied only at protein level, and there is increasing evidence linking minor changes in expression of some modifications with specific diseases.

- While some disorders are known to result from a single gene defect, such as cystic fibrosis (chromosome 7) and sickle cell anaemia (chromosome 11), it is generally accepted that many common diseases such as diabetes, hypertension, deafness, and cancers have more complex causes that may be a combination of sequence variations in several genes - perhaps 20 or many more - on different chromosomes, in addition to environmental factors. It is not possible to identify these genes by sequencing the genome(s) of one, two, or even ten different people – but one can study the proteomes of these individuals to select which 20 or so genes are the important ones.

- In many human diseases, what leads to disease is an incorrect modification or conformation of a normal protein (for example, in protein-folding-related diseases like Alzheimer's, Parkinson's, new-variant CJD, and type II diabetes). Such modifications cannot be seen in or deduced from the genome.

- From a practical standpoint, proteins are almost always useful for disease diagnosis, and the targets of nearly all drugs used in disease therapy are proteins. In order to design the most efficient drug for any disease, one has to find the right target. The best way to do this is to determine all the forms that an individual protein can take, all the proteins with which it interacts, and all the pathways in which it participates.

How do we study a proteome?

Although DNA micro-arrays enable us to view a genome-wide number of active gene products simultaneously in the form of mRNAs, there is often no direct relationship between the *in vivo* concentration of an mRNA and the level of its encoded protein. Differential rates of mRNA translation into protein and differential rates of protein degradation *in vivo* are two factors that confound the extrapolation of mRNA levels to protein expression profiles. Additionally, micro-array analysis is unable to detect, identify, or quantify post-translational protein modifications, which often play a key role in modulating protein function.

Proteomics comprises all comprehensive, high-throughput methods enabling us to display and identify the largest possible number of proteins in a proteome, and to determine how they relate to each other though changes in expression levels or PTMs in response to specific variations in the environment or according to the state of the system under study (i.e. organ, tissue, cell, organelle, micro-organism, or protein complex).

The various techniques used to study the proteome are not as straightforward as those used in transcriptomics, and they span various aspects of protein function:

Structural proteomics is the large-scale analysis of protein structures.
This is achieved using technologies such as high-throughput automated protein expression systems combined with X-ray crystallography and NMR spectroscopy. Structural proteomics also includes extensive *in silico* comparisons and analyses of protein primary and tertiary structures deposited in the

various databases or deduced from genome sequences, with a view to exploring common structural motifs and how they relate to diverse protein functions. Structural analysis can contribute to identifying the functions of newly discovered genes or to showing where drugs bind to proteins or where proteins interact with each other.

Interaction proteomics is the large-scale analysis of protein interactions.
One of the best ways to determine the function of a newly discovered protein is to identify with which molecules it interacts or associates. All classical protein isolation and fractionation techniques (centrifugation, chromatography...) and other technologies such as tandem affinity purification, mass spectrometry, phage display, and the yeast two-hybrid system can be used to isolate protein complexes (for example membrane translocation complexes, ribosomal complexes, transcriptome, spliceosome, nucleosome, respiratory, or photosynthetic complexes) in order to determine protein functions and to study how and why proteins assemble into larger complexes.

Expression proteomics is the large-scale analysis of protein expression and function.
The goal here is to detect and identify all - or a subset - of the proteins present in a particular sample (e.g. a cell, a bacterium, an organelle, or an isolated protein complex) and find out which of these proteins are present, absent, or differentially expressed in a related sample subject to a specific variation. A protein found only in a diseased sample may prove to be a useful drug target or diagnostic marker ("biomarker"). Methods such as two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) or multidimensional liquid chromatography are generally used to separate proteins or peptides in a complex mixture. Following separation, proteins are identified by mass spectrometry combined with protein database searches carried out with appropriate software algorithms.

Protein and antibody micro-arrays are still under development. They may hold enormous potential for proteomic studies. At present, however, their use is far from widespread, and some technological details remain to be dealt with before they become a robust and reliable platform for research and diagnostics.

One of the main challenges encountered in proteomic studies is due to the huge dynamic range of protein expression. In human plasma, for instance, 10 orders of magnitude in concentration separate albumin from the rarest proteins now measured clinically. The difference is expected to reach 12 orders of magnitude in certain proteomes. Under such circumstances high-abundance proteins, sometimes referred to as "housekeeping" proteins, can severely interfere with the detection and profiling of proteins present in low abundance, which are often the interesting ones to study (i.e. transcription factors, kinases, membrane receptors...).

The preparation of a well-defined proteome sample is the basis of any successful proteomic study. Problems arise from the difficulty in preparing or displaying a sample representative of a chosen proteome because of the inherent characteristics of some proteins (poor extraction and/or solubilisation of hydrophobic membrane proteins, very acidic or basic proteins, very large or small proteins).

These considerations drive the effort to design novel proteomics instrumentation and methodology. First, the problem of sample complexity can be addressed by the use of defined reagents and extraction methods and specialised prefractionation techniques for isolating a particular proteome subset. Secondly, increased sensitivity and a wide dynamic range are particularly important for the instrumentation used in detection.

Despite these challenges, proteomics has a tremendous contribution to make towards understanding biological functions and designing better drugs and diagnostics. It is thus expected to drive much of the growth in life science research and instrumentation in the next 5 to 10 years.

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Commentary

Beyond the genome to tissue proteomics

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Full text

The ongoing revolution in molecular medicine can be divided into three phases. The first phase is gene discovery, in which the tools of molecular biology are applied to identify and sequence previously unknown genes. Identification of most of the expressed human genes will be accomplished before 2005. The second phase is molecular fingerprinting, which correlates the genomic state, the complementary DNA expression pattern, and the protein repertoire with the functional status of the cells or tissue. The promise of this phase is that expression profiles can uncover clues to functionally important molecules, and will generate information to tailor a treatment to the individual patient. The third phase is the synthesis of proteomic information into functional pathways and circuits in cells and tissues. This must take into account the dynamic state of protein post-translational modifications and protein-protein or protein-DNA interactions. Through an integrated genomic/proteomic analysis, the ultimate outcome will be an actual functional understanding of the molecular events that underlie normal development and disease pathophysiology. This higher level of functional understanding will be the basis for true rational therapeutic design.

Progress in these three phases of molecular medicine is largely driven by new technologies. The development of polymerase chain reaction, high throughput sequencing, and bioinformatics has been a driving force in the first phase. In the second phase, microhybridization arrays applied to genetic analysis and gene expression [1] is a powerful new tool that has entered the commercial sector, and is becoming widely available to researchers. As more genes are identified, it is likely that specialized arrays will be offered that are specific for a tissue type (eg mammary gland chip), physiologic process (eg apoptosis chip, angiogenesis chip, invasion chip) or class of genes (eg suppressor gene chip, oncogene chip).

Whereas DNA is an information archive, proteins do all the work of the cell. The existence of a given DNA sequence does not guarantee the synthesis of a corresponding protein [2,3]. The DNA sequence is also not sufficient to describe protein structure, function, and cellular location. This is because protein complexity and versatility stems from context-dependent post-translational processes such as phosphorylation, sulfation, and glycosylation. Moreover, the DNA code does not provide information about how proteins link together into networks and functional machines in the cell. In fact, the activation of a protein signal pathway causing a cell to migrate, die, or initiate division can immediately take place before any changes occur in DNA/RNA gene expression. Consequently, the technology to drive the molecular medicine revolution into the third phase is emerging from protein analytic methods.

The term 'proteome', which denotes all the proteins expressed by a genome, was first coined in late 1994 at the Siena two-dimensional gel electrophoresis meeting [4]. Proteomics is proclaimed as the next step after genomics. A goal of investigators in this exciting field is to assemble a complete library of all of the proteins. Only a small percentage of the proteome has been cataloged to date [2,3]. Because 'polymerase chain reaction for proteins' does not exist, sequencing the order of 20 possible amino acids in a given protein remains relatively slow and labor intensive, compared with nucleotide sequencing. Although a number of new technologies are being introduced for high throughput protein characterization and discovery [3,5], the mainstay of protein identification continues to be two-dimensional gel electrophoresis. Two-dimensional electrophoresis can separate proteins by molecular weight in one dimension and charge in the second dimension. When a mixture of proteins is applied to the two-dimensional gel, individual proteins in the mixture are separated out into signature locations on the display, depending on their individual size and charge. Each signature is a 'spot' on the gel, which can constitute a unique single protein species. The protein spot can be procured from the gel and a partial amino acid sequence can be read. In this manner known proteins can be monitored for changes in abundance under treatment or new proteins can be identified. An experimental two-dimensional gel image can be captured and overlayed digitally with known archived two-dimensional gels. In this way it is possible to immediately highlight proteins that are differentially abundant in one state versus another (eg tumor versus normal, or before and after hormone treatment).

Two-dimensional gels have traditionally required large amounts of protein starting material, equivalent to millions of cells. Thus, their application has been limited to cultured cells or ground-up heterogeneous tissue. Not unexpectedly, this approach does not provide an accurate picture of the proteins that are in use by cells in real tissue. Tissues are complicated structures composed of hundreds of interacting cell populations in specialized spatial configurations. The fluctuating proteins expressed by cells in tissues may bear little resemblance to the proteins made by cultured cells that are torn from their tissue context and reacting to a new culture environment. Proteins extracted from ground-up tissue will represent an averaging-out of proteins from all of the heterogeneous tissue subpopulations. For example, in the case of breast tissue the glandular epithelium constitutes a small proportion of the tissue; the vast majority is stroma and adipose. Thus, it has previously been impossible to obtain a clear snapshot of gene or protein expression within normal or diseased tissue cell subpopulations.

To address the tissue-context problem, new technology is again coming to the rescue; creating 'tissue proteomics' as an exciting expanding discipline. Two major technologic approaches have been successfully used to sample macromolecules

directly from subpopulations of human tissue cells. The first technology is laser capture microdissection. This is a technology for procuring specific tissue cell subpopulations under direct microscopic visualization of a standard stained frozen or fixed tissue section on a glass microscope slide. This technology was invented at the US National Institutes of Health and is commercially available through Arcturus Engineering (Mountain View, CA, USA; www.arctur.com). Tissue cells procured by laser capture microdissection have been used for highly sensitive and reproducible proteomic analysis using two-dimensional gels and other analytic methods [6,7,8].

A second major approach to isolate tissue cell subpopulations is affinity cell sorting of dissaggregated cells from pieces of fresh tissue. A highly notable application of this technology in the field of breast physiology was recently reported [9] in a study resulting from a collaboration between Oxford Glycosciences (Oxford, UK) and the Ludwig Institute (London, UK). In that study the investigators separated and purified normal human breast luminal and myoepithelial tissue from reduction mammoplasty specimens using double antibody magnetic affinity cell sorting and Dynabead magnetic sedimentation (Dynal Inc, UK). After using enzymatic treatments and various incubation, separation, and washing steps, the investigators obtained purified luminal and myoepithelial cells in yields of 5×10^6-2×10^7. Proteins from these cell populations were then analyzed using two-dimensional gels. A master image for each cell type comprising a total of 1738 distinct proteins was derived. The investigators found 170 protein spots that were elevated twofold or more between the two populations. Of these, 51 were further characterized by tandem mass spectroscopy. The proteins preferential to the myoepithelial cells contained muscle-specific enzymes and structural proteins consistent with the contractile muscle-related derivation of these cell types.

Myoepithelial cells are a fascinating component of breast tissue. They are thought to play important roles in duct and lobule growth, matrix architecture, and remodeling after lactation and involution. A pathologic hallmark of early cancer progression from carcinoma *in situ* to invasive cancer is the loss or redistribution of myoepithelial cells. The conspicuous absence of myoepithelial cells in breast cancer progression could mean that these cells produce suppressor proteins that normally keep the malignant cells in check. Thus, one or more of the proteins identified in the study by Page *et al* [10] could be candidate cancer prevention molecules. The authors of that study concluded that 'These observations demonstrate that proteomics has the refinement and sensitivity to find proteins that are either uniquely or differentially expressed between different cell types, the consequences of which could enable new strategies for drug discovery.'

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Examiner 109
reference 7

Fibronectin Fragments Modulate Human Retinal Capillary Cell Proliferation and Migration

Maria B. Grant, Sergio Caballero, David M. Bush, and Polyxenie E. Spoerri

Capillary morphogenesis involves cell-cell and cell-matrix interactions. Proteases elaborated by capillary cells modify the extracellular matrix (ECM) to facilitate capillary tube formation. Previously, we detected the presence of fibronectin fragments (Fn-f) associated with the proform of matrix metalloprotease-2 (MMP-2) in conditioned medium of human retinal endothelial cells (HRECs). Association of this fragment to latent MMP-2 prevented autocatalytic activation of MMP-2, suggesting a modulatory role of Fn-f in MMP-2 activation. In this report, we examined the potential role of Fn-f on two processes involved in angiogenesis, proliferation and migration of vascular cells. The effects of Fn-f on proliferation were determined by DNA synthesis and cell counts. Their effects on migration were assessed using modified Boyden chambers. Seven Fn-f were tested on vascular cell migration and/or proliferation. Three Fn-f induced migration. Fn-f of 30-kDa and 120-kDa size positively affected proliferation of microvascular cells but not macrovascular cells. A 45-kDa gelatin binding fragment of Fn inhibited HREC proliferation but stimulated pericyte and smooth muscle cell proliferation. The potency of these fragments exceeded that of the known angiogenic growth factor, basic fibroblast growth factor (bFGF), on HREC migration. ECM components such as fibronectin may influence capillary morphogenesis by the generation of fragments that can modulate proliferation, migration, and protease activation. In the setting of diabetes, excess Fn is generated and is available for degradation. Thus, the production of Fn-f may be specifically relevant to the angiogenesis observed in proliferative diabetic retinopathy. *Diabetes* 47:1335–1340, 1998

Intrinsic to angiogenesis is the migration, proliferation, and formation of capillary tubes by endothelial cells. Quiescent endothelial cells become activated by soluble mitogens and insoluble extracellular matrix (ECM) molecules producing proteases for the degradation of matrix proteins to facilitate capillary tube formation (1). Fibronectin (Fn), a high-molecular-weight, adhesive, multifunctional glycoprotein and a key ECM component, has diverse biological activities (2). Fn exerts growth factor and differentiated activities in many types of cells and plays a vital role in cellular adhesion and migration, oncogenic transformation, wound healing, and hemostasis (3).

One of the most important functions of Fn is the maintenance of normal cell morphology via organization of cell attachment to the ECM. This is accomplished by a series of binding domains, including fibrin, factor XIIa, gelatin/collagen, DNA, heparin, and cell binding domains. The regions between these domains are highly susceptible to proteolysis, which gives rise to fibronectin fragments (Fn-f). Fn-f have been found to have activities not found in the intact molecule, and selected Fn-f have been shown to affect proliferation (4) and stimulate migration (5,6).

In a previous study, we found that human retinal endothelial cells (HRECs) of diabetic origin and nondiabetic HRECs after exposure to glucose expressed a novel proteolytic activity that migrated at 90 kDa. This 90-kDa activity represented the matrix metalloprotease-2 (MMP-2) tightly associated with Fn-f, and association of this fragment inhibited the autoactivation of MMP-2 (7). In the present study, the effect of selected Fn-f on vascular cell proliferation and migration was examined. This study supports that the generation of Fn-f may regulate microvascular cell behavior and that abnormal Fn-f formation in vivo could facilitate aberrant angiogenesis, as seen in proliferative diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Cell cultures. Human eyes were obtained from the National Disease Resource Interchange within 36 h of death. The eyes were dissected, and the retinas were removed and digested. HREC cultures were prepared and the purity of the culture assessed as previously described (8). HRECs were routinely seeded at 6×10^3 cells/cm^2 in 75-cm^2 flasks. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air.

In certain studies, cells were treated with whole Fn (cellular Fn; Sigma, St. Louis, MO) from human foreskin fibroblasts or with the following Fn-f: 30-kDa tryptic (Sigma), which spans a small segment of the terminal end of the heparin II/fibrin I domain and continues into the collagen/gelatin binding domain; 40-kDa chymotryptic (Life Technologies, Gaithersburg, MD), comprising a small segment of the COOH-terminal end of the heparin I/cell binding domain and continues into the heparin III binding domain; 45-kDa tryptic (Sigma), which comprises most of the collagen/gelatin binding domain; 70-kDa cathepsin D (Sigma), which contains most of the heparin II/fibrin I as well as the collagen/gelatin binding domains; 110-kDa chymotryptic (Upstate Biotechnology, Lake Placid, NY), containing the cell binding (but not heparin I) domain; and 120-kDa chymotryptic (Life Technologies), which spans a small portion of the collagen/gelatin binding domain and nearly all of the heparin I/cell binding domain. All fragments were purified by high-performance liquid chromatography, reconstituted according to the manufacturer's instructions, and stored at −80°C in single-use aliquots. All fragments were analyzed by SDS-PAGE and confirmed as single bands by silver staining. To further confirm purity of Fn-f, NH_2-terminal sequencing was performed on the 120-kDa, 45-kDa, and 30-kDa proteins by the Protein Sequencing Core at the University of Florida.

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BFGF, basic fibroblast growth factor; BrdU, bromodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; Fn, fibronectin; Fn-f, fibronectin fragment(s); HREC, human retinal endothelial cell; HUVEC, human umbilical vein endothelial cell; MMP-2, matrix metalloprotease-2; SMC, smooth muscle cell; tPA, tissue-type plasminogen activator.

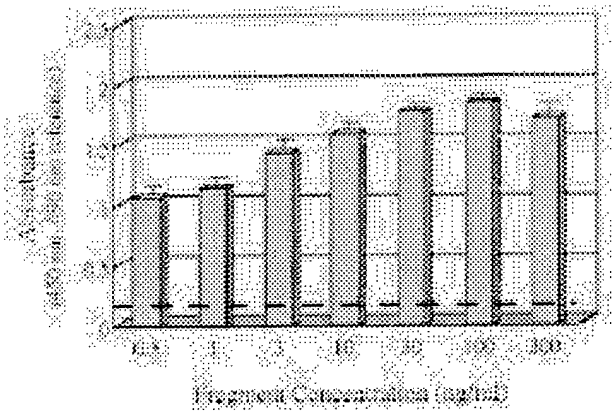

FIG. 1. HREC proliferation in response to increasing doses of the 120-kDa tryptic Fn-f. Cells were made quiescent by withdrawing serum for 18 h before the addition of 120-kDa Fn-f at the doses indicated. After an additional 24 h, the last 4 of which BrdU labeling reagent was added, the cells were processed according to manufacturer's directions to measure BrdU incorporation. Data are expressed as optical density at the wavelength indicated. The dashed line denotes the mean absorbance of untreated cells. Each bar is the mean of quadruplicate determinations, with standard error indicated by the error bar. BrdU incorporation reaches a plateau at 100 ng/ml (P < 0.05 for each dose up to 100 ng/ml vs. the previous dose; NS for 300 vs. 100 ng/ml).

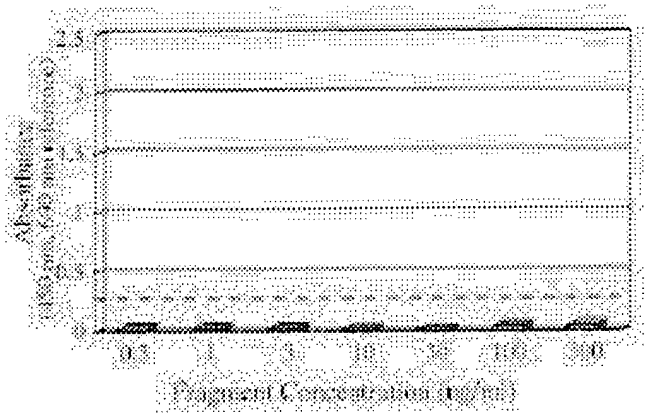

FIG. 2. Response of HUVECs to increasing doses of the 120-kDa chymotryptic Fn-f. These cells and the HRECs were treated identically. Data are expressed as optical density at the wavelength indicated. The dashed line denotes the mean absorbance of untreated cells. Each bar is the mean of quadruplicate determinations, with standard error indicated by the error bar. There is no dose-dependent trend in BrdU incorporation in these cells.

Human coronary artery smooth muscle cells (SMCs) were isolated and cultured as described (9). Human retinal pericytes were isolated at the time of HREC isolation and cultured separately as described (10). Human umbilical vein endothelial cells (HUVECs) were kindly provided by Dr. T. Maciag.

Proliferation. Bromodeoxyuridine (BrdU) incorporation (Boehringer Mannheim, Indianapolis, IN) was used to measure cell proliferation in response to various doses of Fn-f in HRECs, HUVECs, SMCs, and pericytes. Cells were grown in 96-well microtiter plates to ~50% confluence. They were then exposed to each Fn-f at the described doses for the indicated time of either 12 (HREC only), 24, 48, or 72 h. All treatments were performed in triplicate. Proliferation was indicated by a change in absorbance after reaction with anti-BrdU antibody and a colorimetric substrate reaction, as outlined by the manufacturer, and reported as fold change versus untreated cells. Based on the results of these studies, three Fn-f (30, 45, and 120 kDa) were selected for further examination as described below.

Migration studies. Chemotaxis studies were performed as previously described using modified Boyden chambers (11). For these studies, 25 µl of a suspension of HREC (1.6×10^6 cells/ml) was placed in each well of the inverted blindwell apparatus containing 48 wells. Wells were overlaid with a porous (5 µm diameter pores) polyvinyl-free and pyrrolidine-free polycarbonate membrane (Nuclepore, Pleasanton, CA), coated with 40 µg/ml bovine dermal collagen (Sigma). After allowing the cells to adhere to the membrane, chambers were then placed upright and test substances added (50 µl/well). Each Fn-f or whole Fn was tested at the indicated concentration. Dulbecco's modified Eagle's medium (DMEM) served as negative control, and DMEM containing 10% fetal calf serum served as positive control to assess chemotaxis. Chemokinesis, the nonoriented increase in cell locomotion in response to a stimulus, was measured for each Fn-f by adding equal concentrations of each fragment being tested to both upper and lower wells to abolish the concentration gradient. Chambers were disassembled after the specified time, cells on the attachment side were scraped off, and membranes were stained for analysis as previously described (11). Cells that migrated through the membrane were counted for each well and reported as cells per high power field. Additional chemotaxis experiments were performed comparing the effect of eqimolar concentrations of Fn-f and basic fibroblast growth factor (bFGF), since bFGF is a known stimulator of migration.

RESULTS

Proliferation. Of the five Fn-f examined, only three affected proliferation in HRECs. The 30-kDa and 120-kDa fragments induced proliferation as assessed by increased BrdU incorporation and cell number. Addition of the 120-kDa Fn-f resulted in a dose-dependent increase in BrdU incorporation in HRECs. Even at the lowest concentration examined, there was a 40-fold increase over basal, reaching maximal effect at 100 ng/ml (Fig. 1). When HUVECs were exposed to varying concentrations of the 120-kDa fragment, no change in BrdU incorporation was observed at any concentration tested (Fig. 2). The 30-kDa fragment resulted in a dose-dependent increase in BrdU incorporation. The maximal effect was achieved at 10 ng/ml. Higher concentrations resulted in stimulation, but not as great as at 10 ng/ml (Fig. 3). The 45-kDa fragment inhibited BrdU incorporation in HREC compared with untreated HREC (Fig. 3). However, the 45-kDa Fn-f increased BrdU incorporation in a dose-dependent manner in SMCs and pericytes (Fig. 4*A* and *B*). A maximal six- to sevenfold increase in BrdU incorporation was observed with both pericytes and SMCs, in contrast to the 20-fold decrease seen with this fragment in HRECs. The 30-kDa and the 120-kDa fragments increased BrdU incorporation

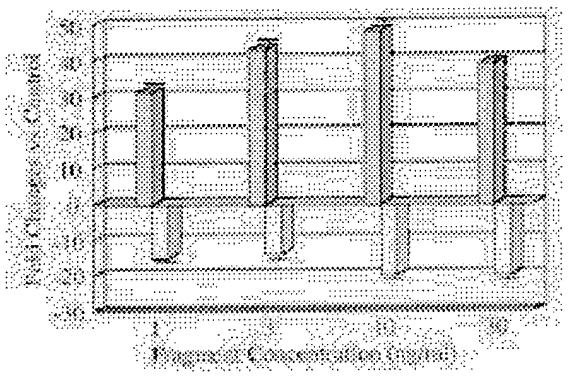

FIG. 3. HREC proliferation in response to increasing doses of the 30-kDa tryptic or 45-kDa tryptic Fn-f. Cells were treated identically to HRECs exposed to the 120-kDa Fn-f. Data were normalized to untreated cells and expressed as fold change. Each bar is the mean of quadruplicate determinations, with standard error indicated by the error bar. The 30-kDa fragment (▨) exhibits a dose-dependent increase in BrdU incorporation that reaches a plateau at 30 ng/ml (P < 0.05 for each dose up to 10 ng/ml vs. the previous dose; NS for 30 vs. 10 ng/ml). The 45-kDa fragment (□) exhibits a dose-dependent decrease in BrdU incorporation in these cells (P < 0.05 for each dose up to 30 ng/ml vs. the previous dose).

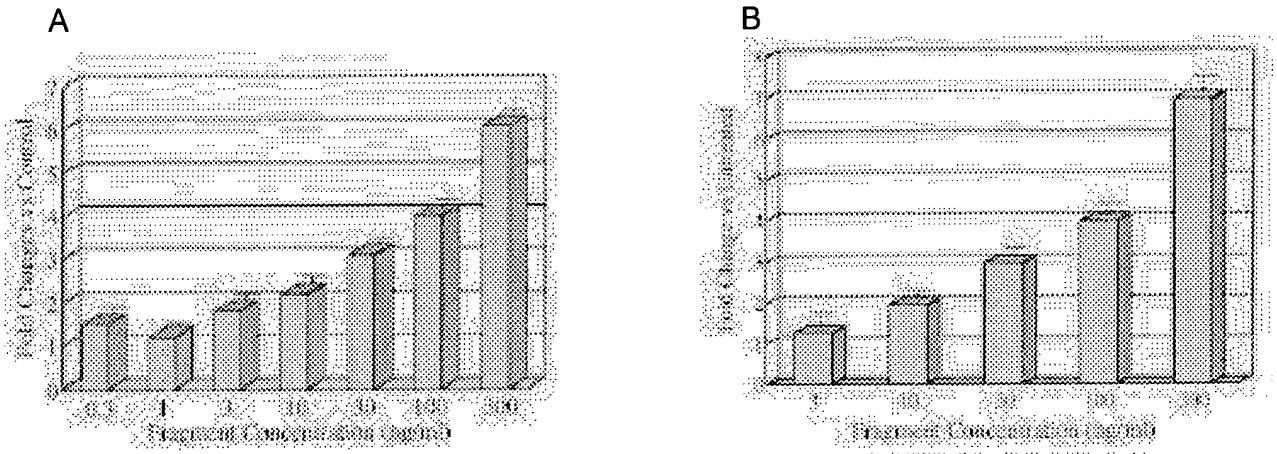

FIG. 4. Effect of increasing doses of the 45-kDa tryptic Fn-f on SMCs (*A*) or pericytes (*B*) in culture. Cells were treated identically to HRECs exposed to the 120-kDa Fn-f. Data were normalized to untreated cells and expressed as fold change. Each bar is the mean of quadruplicate determinations, with standard error indicated by the error bar. *A*: The 45-kDa Fn-f induces DNA synthesis in cultured SMCs in a dose-dependent fashion, showing a sixfold change versus untreated cells at the highest dose tested ($P < 0.05$ for each dose from 30 to 300 ng/ml vs. the previous dose; NS for 0.3 vs. 1 ng/ml). *B*: Cultured pericytes also show a dose-dependent increase in DNA synthesis in response to the 45-kDa Fn-f, with a sevenfold increase at the highest dose tested ($P < 0.05$ for each dose vs. the previous dose).

two- to threefold in SMC (data not shown) in contrast to the 35- to 70-fold increases observed in HREC.

The effect of exposure to Fn-f on cell number in HREC was examined. Cell number at 24 h rose in a dose-dependent manner by the addition of the 30-kDa and 120-kDa fragments. The addition of the 45-kDa fragment resulted in a dose-dependent decrease in cell number. At the highest concentration of 45-kDa Fn-f examined, cell number fell below that of wells containing medium alone (Fig. 5). The proliferative effect was evaluated only at 24 h exposure, because at 48–72 h the autocrine production of growth factors by these cells makes interpretation of the effect of the Fn-f difficult. The 120-kDa fragment and 30-kDa fragment did not stimulate an increase in cell number at 24 h in HUVEC (data not shown).

Migration studies. The three fragments examined above were tested in modified Boyden chambers. The 30-kDa fragment showed a dose-dependent increase in HREC migration (Fig. 6*A*). Similar results were observed with the 120-kDa fragment (Fig. 6*B*). The 45-kDa fragment also induced migration in a dose-dependent fashion (Fig. 6*C*). The chemotactic response observed with the 120-kDa fragment and the 45-kDa fragment exceeded the response observed with equal molar concentrations of intact Fn, whereas the effect of Fn on HREC migration was not significantly different from that observed with the 30-kDa Fn-f (data not shown). Interestingly, the response observed with the 120-kDa and 45-kDa Fn-f was greater than the response observed with equal molar concentrations of bFGF (Fig. 6*B* and *C*). Also, the fragments induced significant amounts of migration at 4 h of exposure, whereas the bFGF response required 8 h of exposure for optimal response in this system (data not shown). The chemokinetic effect of the 30-kDa, 45-kDa, and 120-kDa fragments was evaluated by checkerboard analysis and was not significant.

DISCUSSION

Capillary morphogenesis involves the orchestrated effects of growth factors that modulate endothelial cell proliferation, migration, and tube formation. Mesenchymal precursors are recruited to encase the endothelial tube. The precursors differentiate into pericytes, and there is inhibition of endothelial proliferation, resulting in a mature capillary. Growth factors and ECM proteins can modulate each of these steps in capillary formation.

The ECM protein Fn is present in the walls of vessels, is concentrated at pericyte endothelial contacts (12), and is also present in the internal limiting membrane of the adult retina. Although not all studies have been able to detect Fn in the basement membrane of capillaries, the inability to do so may have been due to the type of fixation used (12), to the fact that postembedding immunogold techniques were performed (13), or to the fact that antigen retrieval techniques needed for many basement membrane proteins were not used. Others who did find Fn used a variety of more sensitive

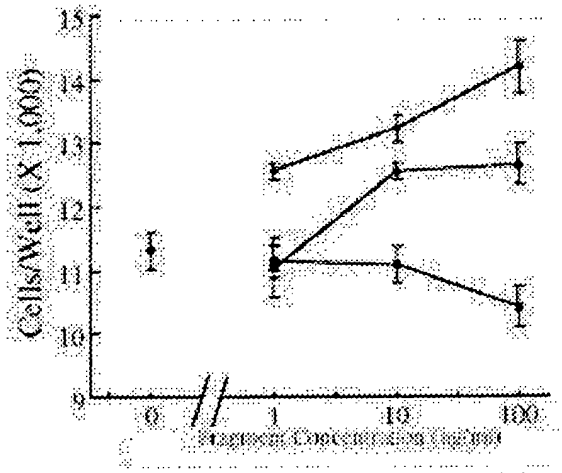

FIG. 5. Effect of 30-kDa tryptic (▲), 45-kDa tryptic (■), and 120-kDa chymotryptic (●) Fn-f on HREC proliferation as measured by change in cell number. Cells were plated in 24-well multiwell plates, then made quiescent by withdrawing serum for 18 h before adding Fn-f at the doses indicated. Serum-free medium was used as negative control (◆). After an additional 24 h of incubation, the cells were enzymatically dissociated and each well counted separately using a Coulter counter. Each point is the mean of four determinations. Error bars show standard error. Both the 30-kDa and 120-kDa Fn-f induce cell proliferation, whereas the 45-kDa fragment inhibits proliferation.

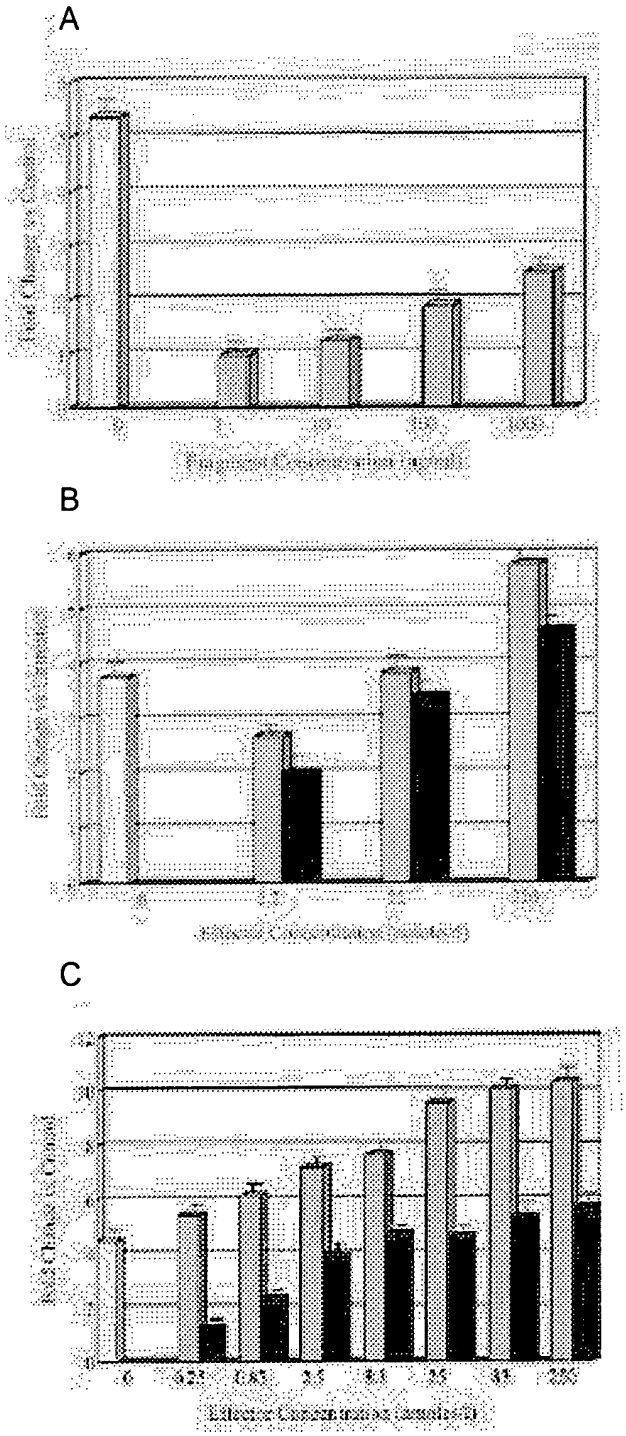

FIG. 6. Effect of Fn-f on migration of HREC, as measured using modified Boyden chambers. All three fragments induced dose-dependent migration of cultured microvascular endothelial cells. In all cases, medium containing 10% (wt/vol) fetal bovine serum was used as positive control. Each bar represents the mean of eight determinations, with error bars denoting standard error. All data were normalized to unstimulated cells (media alone) and expressed as fold change in the number of cells migrating. *A*: The 30-kDa fragment induces up to a 2.5-fold increase in migration at the highest dose tested, about half that induced by media + fetal bovine serum (□ on all three parts). The dose-dependent migration induced by either the 45-kDa (*B*, ▨) or the 120-kDa (*C*, ▨) fragments is actually greater (at maximally tested doses) than that induced by either media + fetal bovine serum or equal molar concentrations of bFGF (*B* and *C*, ■). In all cases, the data represented by each bar are significantly different ($P < 0.05$) from the previous dose.

techniques, including immunofluorescence and immunoperoxidase (14,15).

Immunofluorescence studies after trypsin digestion of the retina showed increased Fn immunoreactivity in large vessels and microvessels of patients with diabetes compared with control subjects. In situ hybridization studies of the trypsin-digested retinas from these diabetic patients showed Fn mRNA in the retina and retinal microvessels, a direct argument in support of local synthesis of Fn in the vessels of the human retina (16).

Using electron microscopy immunogold studies, we observed increases in Fn immunoreactivity in capillary basement membranes of rats with spontaneous diabetes compared with age-matched nondiabetic controls (17). In addition, the basement membrane zone of many new blood vessels is positive for an oncofetal Fn isoform containing the ED-B domain, a marker of angiogenesis (18,19).

Previous studies from our laboratory have shown that HRECs of diabetic origin expressed increased amounts of Fn compared with HRECs of nondiabetic origin. Exposure of HRECs of nondiabetic origin to high glucose increased the amount of Fn protein expressed (20). The diabetes-induced overexpression of Fn by endothelial cells is not readily reversible, and high glucose can mimic this effect in endothelial cells of nondiabetic origin. After six or seven cell replications, the glucose-induced elevation in Fn and type IV collagen is greater than in control cells (21). The differences we observed in HRECs of diabetic and nondiabetic origin give support to the finding that the events occurring during a finite period of metabolic derangement can leave long-lasting sequelae in the system (8). Processes that could propagate a "memory" of the diabetic state include hyperglycemia-induced irreversible modifications of long-lived ECM proteins. Although some effects of diabetes can be reversed by adequate insulinization, Fn overexpression and increased synthesis of glomerular basement membrane collagen do not decrease with treatment of diabetes. Increased Fn synthesis is also observed in fibroblasts explanted from diabetic mice and passaged in culture (22).

Proteolysis of Fn occurs near cells undergoing neoplastic transformation (23). Tumor cells elaborate proteases that can cleave Fn, including plasmin (24–26). Fn-f have been identified at sites of inflammation, injury, and destruction by metastatic tumor cells (27–31). Fn-f have been found to have activities not found in the intact molecule. Selected Fn-f have been shown to affect proliferation (27,28) and promote the adhesion (30,31), spreading, and migration of vascular endothelial cells (4–6,31,32).

Fn-f also induce expression of various proteases, including elastase (33), stromelysin (34), and metalloproteases (35,36). Studies by Imhoff et al. (37) demonstrated that a 190-kDa fragment of Fn produced by cathepsin D proteolysis in the presence of Ca^{2+} undergoes spontaneous autolysis, generating two enzymes, Fn-gelatinase and Fn-laminase, specific for the degradation of the ECM proteins, laminin, and Fn.

Recent studies have shown that Fn-f derived from residues 196–203 are potent stimulators of plasminogen activation catalyzed by tissue-type plasminogen activator (tPA) (38). Fn-f increased the efficacy of the plasminogen substrate. This region of Fn was within the fifth type-1 repeat in the NH_2-terminal domain of Fn. The primary physiological role of type-1 repeats is to bind fibrin, which

enhances the catalytic activity of tPA. Thus, interaction of plasminogen and tPA with ECM components may provide a fine regulatory mechanism for localized generation of plasmin proteolytic activity within the ECM.

Previous studies in our laboratory showed that HRECs express tPA in the quiescent state and urokinase when wounded in culture, and that plasmin activity was easily detectable in the conditioned medium of HRECs under basal conditions (8). The generation of plasmin can lead to activation of proforms of MMPs, and plasmin can degrade Fn, as can MMP-2. We have recently shown that HRECs produce MMP-2 (7). Fn-f could be generated in vivo at sites of angiogenesis by proteases secreted by endothelial cells (e.g., plasmin and MMPs) and/or mast cells (e.g., tryptase) (39).

The present study shows that the 120-kDa fragment increases DNA synthesis and cell number, but only in capillary endothelial cells, as this effect was not observed in HUVEC. The effect of the 30-kDa Fn-f is also specific to endothelial cells of the microvasculature, as the fragment did not affect HUVEC proliferation. We observed that selected fragments (45-kDa Fn-f) had opposite effects on HRECs and pericytes, a response that would be particularly beneficial if inhibition of endothelial cell proliferation was desired at the same time pericyte proliferation was required. For example, during the final stages of angiogenesis, the endothelial tube is already formed, but pericytes must still migrate and proliferate to encase this endothelial tube to complete the formation of the capillary. We have also observed that the Fn-f are more potent and act more quickly than bFGF on endothelial cell migration.

In summary, this study and previous work from our laboratory provide unique evidence for specific Fn-f regulating both cell movement and cell proliferation and activation of latent MMP-2 (7). Fn and Fn-f have potentially competing actions on steps relevant to angiogenesis. These studies support a complex regulatory role of Fn and its various domains, obtained by proteolytic degradation of Fn, in angiogenesis. The modulation by glucose of ECM components, such as Fn, may modify cellular behavior that may be specifically relevant to angiogenesis in diabetic retinopathy.

ACKNOWLEDGMENTS

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Gluconeogenesis, glucose ...

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Gluconeogenesis, glucose production and fasting glycaemia

Original article:
Influence of obesity and type 2 diabetes on gluconeogenesis and glucose output in humans. A quantitative study. Gastaldelli A, Baldi S, Pettiti M et al. Diabetes 2000; 49: 1367-73.

Summary

Increased endogenous (hepatic and possibly renal) glucose production is thought to be a major factor regulating fasting glycaemia in Type 2 diabetes. Whether it relates to enhanced gluconeogenesis, elevated glycogenolysis, or both, is debatable. Little information is available on the effects of obesity per se on endogenous glucose production.

In the present study, fasting endogenous glucose fluxes and glycaemia were measured in 40 obese subjects (28 with Type 2 diabetes and 12 non-diabetic controls) and 15 non-obese subjects (nine with Type 2 diabetes and six non-diabetic controls). Endogenous glucose production was measured using 6,6-di-deuterated glucose, and gluconeogenesis by the 2H2O technique [1].

Several interesting observations were made. First, gluconeogenesis was increased in obese non-diabetic subjects. Second, gluconeogenesis was markedly increased in both obese and non-obese diabetic subjects. Third, gluconeogenesis and endogenous glucose production were both positively correlated with fasting glycaemia (Fig. 1). And, finally, hyperglucagonaemia was present in diabetic patients and was positively correlated with glucose fluxes.

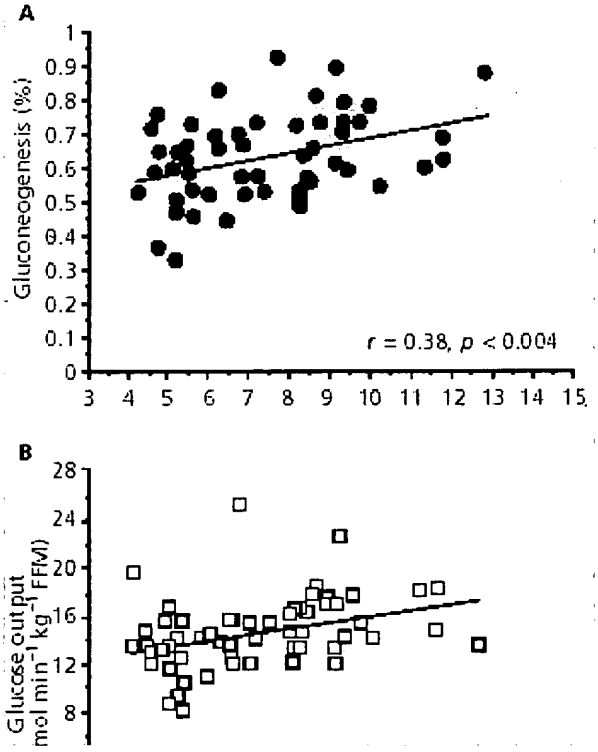

Fig. 1: Linear relationship between fasting plasma glucose and percent gluconeogenesis (A) and endogenous glucose output (B).

Comment

The fact that increased glucose production plays an important role in the pathogenesis of fasting hyperglycaemia in Type 2 diabetes is well documented. However, the mechanisms responsible for this increased glucose production are unknown. Several factors may be involved, including hepatic or renal resistance to the suppressive actions of both glucose and insulin on glucose production, increased secretion of

counterregulatory hormones, intrinsic dysregulation of hepatic/renal pathways of glucose production, or extrahepatic consequences of insulin resistance. Among the latter, increased lipolysis and proteolysis may stimulate gluconeogenesis by increasing gluconeogenic substrate availability and by enhancing plasma free fatty acid concentrations. High plasma free fatty acid concentration may in turn enhance hepatic gluconeogenesis and glucose output [2].

Obesity per se is also characterized by elevated rates of lipolysis and hence of glycerol and fatty acid release by adipose tissue. It may therefore be a major factor in enhanced glucose production under special circumstances. The present study carefully measured glucose production and gluconeogenesis in a large group of subjects including obese and non-obese individuals. The several pitfalls associated with measurements of glucose fluxes were skilfully avoided. The results obtained in obese subjects corroborate our earlier observations, i.e. that obesity per se stimulates gluconeogenesis without altering total glucose output [3, 4]. It has been recognized for several years that stimulation of gluconeogenesis can be attained by infusion of gluconeogenic precursors such as glycerol [5] or lactate [6]. Under such circumstances, an autoregulatory mechanism within the liver appears to prevent an increase in glucose production by simultaneously reducing net glycogenolysis [7]. This study clearly indicates that this autoregulatory mechanism is intact in obese non-diabetic patients.

In obese Type 2 diabetics, the picture, however, was quite different. Gluconeogenesis was further enhanced, possibly related to more severe extrahepatic and hepatic insulin resistance, and resulted in an increased glucose-6-phosphate flux in glucose-producing cells. Furthermore, hyperglucagonaemia was present, and is likely to play a major role in increasing glucose production, possibly by stimulating glucose-6-phosphatase. Last, but not least, both increased glucose production and whole body insulin resistance (documented by lower glucose clearance in diabetic patients) contributed to the development of hyperglycaemia.

What can we learn from these data? First, increased gluconeogenesis is not a sufficient factor per se to produce fasting hyperglycaemia. This suggests that strategies aimed at reducing gluconeogenesis may be ineffective in reducing glycaemia in Type 2 diabetes. This conclusion appears to be supported by the observation that acute ethanol administration did not lower glycaemia, although it suppressed gluconeogenesis in Type 2 patients [8]. Second, additional factors are present in Type 2 diabetes to stimulate glucose production. Hyperglucagonaemia may be one such factor, but other hormones or cytokines as well as neural factors may also be involved. Identification of such factors and of their mode of action may point to novel potential therapeutic strategies to reduce glucose production and fasting hyperglycaemia in Type 2 diabetes.

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Summary and Comment:
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